The synergism of temperature, pH and growth phases on heavy metal biosorption by two environmental isolates

Jingjing Fan,[a] Tugba Onal Okyay, [a] Debora F. Rodrigues [a*]

[a] Department of Civil and Environmental Engineering

University of Houston, Houston, TX 77204-4003 (USA)

Corresponding Author: Debora F. Rodrigues Ph.D.
N136, Engineering Bldg 1
4800 Calhoun Rd, Houston, Tx 77204

E-mail: dfrigirodrigues@ uh.edu
Phone: +1-713-743-1495 Fax: +1-713-743-4260

ABSTRACT

In real environmental applications, such as heavy metal bioremediation, microorganisms are generally not kept at their optimum growth conditions; therefore, it is imperative to investigate their heavy metal removal performance under diverse environmental conditions. The present study aims to investigate the effects of pH, temperature and growth phases on the removal of Cu^{2+} and Cr^{6+} by two environmental isolates identified as *Ochrobactrum* sp. and *Cupriavidus metallidurans* CH34. Results showed that cells in logarithmic phase presented better biosorption capacity than cells in stationary phase for both isolates. The Cr^{6+} metal was removed more efficiently by live *Ochrobactrum* sp. than dead cells; while dead *C. metallidurans* CH34 biosorbed better than live ones. It was also found that the pH and temperature affected the bioadsorption capacity. The optimum temperatures were determined to be 37ºC and 27ºC, and the optimum pH values were 6 and 7 for *Ochrobactrum* sp. and *C. metallidurans* CH34, respectively. Additionally, both microorganisms preferentially adsorbed Cu^{2+} in Cu^{2+}/Cr^{6+} mixtures. The main mechanism of adsorption was determined to be through carboxylic, hydroxyl, and amino functional groups.

KEYWORDS: Copper, chromium, *Cupriavidus metallidurans* CH34, *Ochrobactrum* sp., biosorption

1. INTRODUCTION

Rapid industrialization has led to increase environmental impacts. For instance, effluent discharges from textile, electroplating, and battery industries are commonly contaminated with heavy metals [1]. Unlike organic pollutants, heavy metals are non-biodegradable, accumulate in living organisms via food chain, and some of them are extremely toxic even at relatively low concentrations. Therefore, problems arising from heavy metal pollutions require urgent removal solutions.

For heavy metal removal from water and wastewater, conventional methods, such as chemical reduction and precipitation, have been commonly used. However, they are only effective when the metal concentration is low [2]. Other methods, like ion exchange, membrane technologies and activated carbon adsorption, are expensive and inefficient in terms of energy and chemical consumption, especially at low metal concentrations between 1-100 mg/L [3, 4]. Therefore, economical and efficient alternatives are highly desirable.

Diverse studies have suggested that bacterial biomass can be promising and inexpensive biosorbents for the removal of metal ions [5-7]. Other studies have demonstrated that, depending on the microorganism, dead biomass can be more effective in removing heavy metals than live biomass [8]. However, to the best of our knowledge, detailed comparative mechanistic investigations on heavy metal biosorption by dead and live biomasses under relevant environmental conditions have not been fully assessed. This is the first study, to the best of our knowledge, to compare heavy metal biosorption under optimum microbial growth conditions with environmental relevant conditions that are not necessarily optimal for microbial growth and heavy metal biosorption.

In the present study, we compare two environmental isolates, *Ochrobactrum* sp. and *C. metallidurans* CH34. *Ochrobactrum sp*. is a new environmental isolate obtained from a lysimeter containing organic wastes and heavy metals. *C. metallidurans* CH34 is also an environmental isolate that was selected as a model organism for comparison, because it has been extensively described as one of the best biosorbents [9].

The present study aims to investigate Cu^{2+} and Cr^{6+} bacterial resistance and determine the maximum adsorption capacity of these two microorganisms under different growth phases, pH values and temperatures. Finally, competitive biosorption assays with both Cu^{2+} and Cr^{6+} is also investigated under their optimum microbial growth and under environmental conditions similar to their isolation sites.

2. MATERIAL AND METHODS

2.1. Microorganisms and Growth Conditions

C. metallidurans CH34 was isolated from a sludge of a zinc decantation tank [10] and *Ochrobactrum* sp. was obtained from leachate samples generated by a lysimeter containing organic wastes and heavy metals in Brazil. Prior to each experiment, *C. metallidurans* CH 34 and *Ochrobactrum* sp. were grown on Tryptic Soy Agar (TSA) at 27ºC overnight (Difco) to obtain single colonies. Single colonies for each microorganism was transferred to Tris-medium (TSM), which contained 6.06 g/L base Tris, 4.68 g/L NaCl, 1.49 g/L KCl, 1.07 g/L NH_4Cl, 0.43 g/L Na_2SO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.03 g/L $CaCl_2 \cdot 2H_2O$, 0.23 g/L $Na_2HPO_4 \cdot 12H_2O$, and 0.005 g/L of Fe(III)(NH_4) citrate for the subsequent experiments [11]. The pH of TSM was adjusted to 7.0±0.5 using 1M HCl and 1M NaOH. Sterilization of TSM was achieved by autoclaving at 121ºC for 20 min, and then it was cooled at room temperature. Afterwards, 1 mL of filter-sterilized (0.22µm)

trace element solution was added to the TSM in aseptic conditions [12].

Single colonies of each microorganism were grown in 25 mL of TSM in 50-mL PP centrifuge tubes (Corning Inc., USA) by incubating them at 27ºC and 80 rpm (INNOVA 44, New Brunswick Scientific Co., USA) for 36 h and 48 h to obtain the cells in logarithmic and stationary phases, respectively (Figure S1). In the adsorption experiments, 27ºC and pH 7 were defined as the environmental conditions [13], and the optimum condition will be determined in the optimization section. Dead cells were prepared by autoclaving the 48 h grown cells at 121ºC for 20 min [14]. Afterwards, live or dead cells were harvested by centrifugation at 8000 rpm for 8 min, and washed once with fresh TSM. Finally, the cells were re-suspended in phosphate buffered saline (PBS) (Amresco Inc., USA) to an optical density of 0.3 at the 600 nm (OD_{600}), and the PBS-cell solutions were used in further experiments.

2.2. Determination of Minimum Inhibitory Concentration (MIC)

Copper and chromium heavy metal stock solutions (2000 ppm) were prepared using $CuSO_4 \cdot 5H_2O$ and $K_2Cr_2O_7$ (Fisher Scientific) within TSM. They were filter-sterilized by 0.2 µm PTFE membrane filters (Millipore). Cells grown in TSM broth without heavy metals at 27ºC and 80 rpm for 48 h were used for the MIC experiment. A volume of 20 µL of each PBS-cell suspension was inoculated into a 96-well plate (Corning Inc., USA), which contained 200 µL of TSM having Cu^{2+} (5-1000 ppm) or Cr^{6+} (10-1000 ppm). Negative controls were performed in the same concentrations of heavy metals in TSM without bacteria. Positive controls were prepared by 10% (v/v) inoculation of *C. metallidurans* CH34 or *Ochrobactrum* sp. into heavy metal-free TSM broth. The 96-well plate was incubated in the plate reader (Synergy MX Microplate reader, BioTek,USA) at

27ºC and 80 rpm for 48 h, and every hour absorbance was read at 600 nm in order to determine the MIC. Each experiment was performed in triplicate.

2.3. Assessing the Relative Susceptibilities of Logarithmic- and Stationary-Phases to High Heavy Metal Concentrations

A modified method of Teizel [15] was used to assess the microbial susceptibility on heavy metals. As previously described, PBS-cell solutions in logarithmic- and stationary-phases were inoculated into 100 ppm Cu^{2+} and Cr^{6+} solutions. Cells were exposed to heavy metals for 3 h at 27ºC. The negative controls were also conducted with heavy metal-free TSM. After 3 h of exposure, cellular viability was estimated by the plate count method [13].

2.4. Time-Course Biosorption under Environmental Conditions

The PBS-cell solutions in logarithmic and stationary phases, as described previously, were inoculated into 30 mL TSM containing 100 ppm Cu^{2+} or Cr^{6+} and incubated under environmental conditions for 400 min. Heavy metal solutions without microbes were used as controls to take into consideration any heavy metal precipitation. During the procedure, 3 mL samples were aseptically removed at specific time intervals for 400 min and filtered through 0.2 μm filters. Filtrates were analyzed with the atomic absorption spectrometer (AAS) (Perkin Elmer AAnalyst^{TM} 200). The biosorption capacity (q; mg metal/g dry cell) was calculated using the formula: $q = (C_0 - C_e)/X$, where C_0 and C_e is the initial and the residual metal concentration (mg/L); and X is the biomass concentration (g/L) [7]. In order to calculate the biosorption capacity and represent the dry cell mass of the proposed cell concentration (OD_{600}=0.3), absorbance-dry cell mass curves were obtained (Figure S2).

2.6. Optimization of pH and Temperature for Biosorption

Batch experiments were conducted in metal solutions at pH varying from 3.0 to 8.0 (±0.1). The experiments were all incubated at 27ºC and 80 rpm for 400 min. The effects of different temperatures on the heavy metal sorption by the biomass were determined at pH 7 in the following temperatures: 22^0C, 27^0C, 32^0C, and 37^0C. Heavy metal solutions (100 ppm) were inoculated with PBS-cell solution, as previously described.

In both pH and temperature experiments, aliquots of 3 mL were removed aseptically for 400 min and then filtered. The metal concentrations in the filtrates were analyzed using AAS. Biomass-free heavy metal solutions were also investigated as controls.

2.7. Determination of Adsorption Isotherms

Same amount of cell concentrations of *C. metallidurans* CH34 and *Ochrobactrum* sp. in logarithmic and stationary phases, as well as samples of dead biomasses were suspended in Cu^{2+} or Cr^{6+} solution with concentrations ranging from 10-100 ppm with 10 ppm intervals. Controls were prepared for each metal concentration without biomass. To obtain the isotherms, the adsorption experiments were performed for a period of 400 min under environmental and optimum growth conditions. Experimental data were modeled using the Langmuir isotherm to determine the characteristic parameters (Q_{max} and K_d) of biosorption. The Langmuir isotherm equation used was as follow: $q=Q_{max}C_e/(K_d+C_e)$, where q_{max} represents maximum adsorption capacity (mg/g dry cell), and K_d represents the Langmuir constant (mg/L) [3].

2.8. Competitive Biosorption Assays

Solutions of 30 mL containing both heavy metals at 100 ppm concentration with different ratios (Cu^{2+}:Cr^{6+}; 4:1, 2:2, and 1:4) (v/v) were investigated to determine heavy metal

biosorption preference by the microorganisms. The cell concentrations and the experimental conditions were kept identical to the previous section. The removal ratios of Cu^{2+} and Cr^{6+} were determined by the following equation: removal ratio (%)=[(C_0–C_e)/C_0]×100, where C_0 and C_e are the initial and residual metal concentrations (mg^{-1}) [3].

2.9. Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS) Analyses

SEM and EDS analyses were employed to confirm the presence and identity of the metal ions adsorbed by the biomasses. The microorganisms exposed to heavy metals and the negative controls, which had no exposure to heavy metals, were prepared based on the procedure described by Mejias et al. [16]. After drying the samples overnight at room temperature, they were analyzed with SEM/EDS (JEOL JSM-6010LA) at 15kV of accelerating voltage with an emitting current of 15 µA and 5000X magnification.

2.10. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

Infrared spectra of live and dead biomasses exposed to Cu^{2+} and Cr^{6+} and negative controls were used to characterize the functional groups responsible for adsorbing the heavy metals. After exposing the samples to the heavy metals, 2 mL aliquots were filtered through 0.2 µm filters and dried at room temperature for 48 h. The dry filters were analyzed with the Nicolet iS10 Mid Infrared FT-IR Spectrometer equipped with ZnSe crystal and the Omnic 8 Software (Thermo Fisher Scientific). Infrared spectra were recorded in the range of 4000-400 cm^{-1} with a resolution at 4 cm^{-1}. The background noise of atmospheric water and CO_2 was automatically subtracted from the sample's spectra.

2.11. Statistical Analysis

Each set of experiments was carried out in triplicate. For all results, averages and

standard deviations were calculated in the Microsoft Excel. Further statistical analyses were performed using unpaired t-test to determine statistical difference between the logarithmic phases, stationary phases and the controls.

3. RESULTS AND DISCUSSIONS

3.1. Minimum Inhibitory Concentration (MIC) of Cu^{2+} and Cr^{6+}

The MIC results showed that *C. metallidurans* CH34 was more resistant to Cu^{2+} (750 ppm) than *Ochrobactrum* sp. (300 ppm) (Table 1). *Ochrobactrum* sp., on the other hand, showed a MIC value of 900 ppm higher than *C. metallidurans* CH34 for Cr^{6+}. These microorganisms were isolated from different environments with different levels of heavy metal contaminations, therefore it is expected that they would have different levels of tolerance to different heavy metals as observed in this study. It is also worth pointing out that species from the same genus may have different tolerances to the same heavy metal, such as the *Ochrobactrum* sp. MIC for Cu^{2+}, which was 2.3 times higher than the MIC for *Ochrobactrum intermedium* CrT-1 in a previous study [17]. The variation on the MIC results for Cu^{2+} among the two species might be attributed to the use of different growth medium; since a rich medium was used for *O. intermedium* CrT-1, while we conducted our experiments in a minimum medium [17]. Complexation studies between metal cations and components of growth media showed that MIC values obtained in rich media are usually two to five times higher than in salt-based minimum media [18].

Table 1. MIC for *C. metallidurans* CH34 and *Ochrobactrum* sp.

Strain	MIC (ppm)	
	Cu^{2+}	Cr^{6+}
C. metallidurans CH34	750	100
Ochrobactrum sp.	300	1000

Additionally, this study also showed that both isolated microorganisms, *C. metalliturans* and *Ochrobactrum* sp., presented higher MIC to Cu^{2+} than other well-known microorganisms, such as *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* [19]. In the case of Cr^{6+}, the MIC results for *Ochrobactrum* sp. was considerably higher than for *C. metallidurans* CH34 and other reported studies with other bacteria, such as *Pantoea sp.* [20], and fungi, *e.g. Aspergillus niger* and *Penicillium* sp. [21]. These results suggest that this new isolate is well adapted to survive in environments polluted with high levels of Cu^{2+} and Cr^{6+} and has a higher tolerance for Cr^{6+} than *C. metallidurans* CH34.

3.2. Microbial Survival after Exposure to High Concentrations of Cu^{2+} and Cr^{6+}

The results of the microbial survival after exposure to 100 ppm of Cu^{2+} and Cr^{6+} revealed a 0.5-2.0 log removal when compared to the controls (Figure 1). The results also show that the cells in stationary phase were less susceptible to heavy metals than the cells in the logarithmic phase, since the cells in logarithmic phase were at least 60% less viable than the ones in stationary phase.

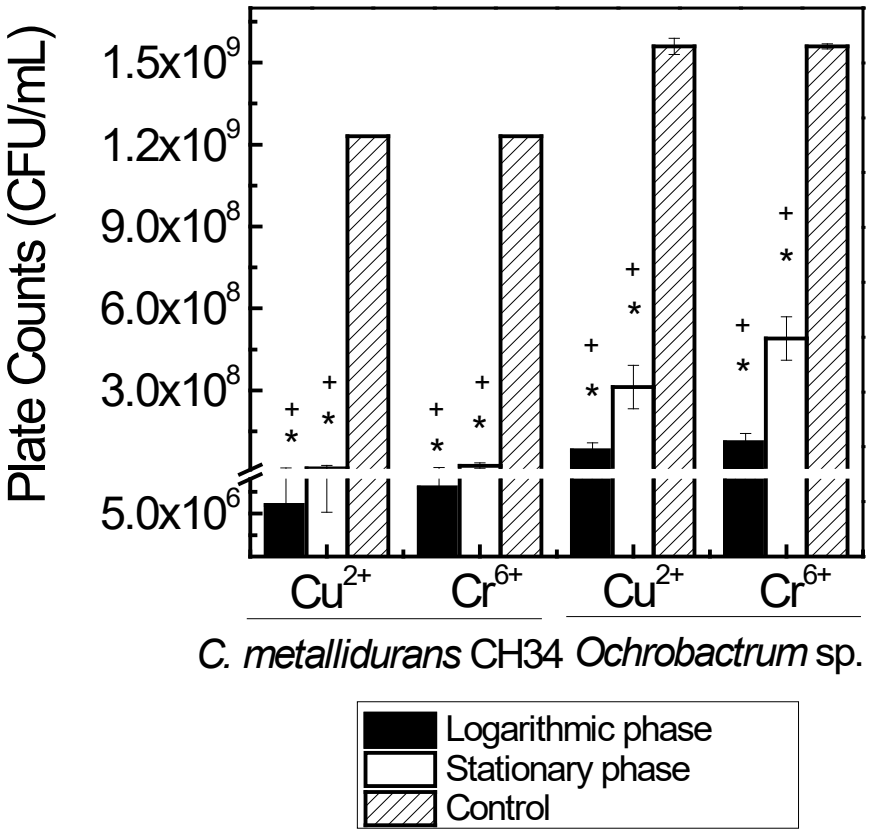

Figure 1: Survival of *C. metallidurans*CH34 and *Ochrobactrum* sp. in different growth phases after exposure to 100ppm of Cu^{2+} and Cr^{6+}. The Symbols * and + correspond to statistically different results between the control and other samples with a 95% confidence interval. Controls have no heavy metals.

The decreased inactivation of cells in stationary phase when exposed to the heavy metals can be explained by the reduced growth rates at that phase, as opposed to the logarithmic phase that presents exponential growth. The reduced growth rates of microorganisms under nutrient starvation have been widely reported to influence microbial susceptibility to antimicrobial agents for a wide range of microorganisms [22]. Additionally, microorganisms in different growth phases can have different membrane lipid compositions, cell metabolism, and cell wall compositions, which would directly affect their susceptibility to toxic chemicals [23]. These phenomena explain the lower

inactivation of *C. metallidurans* CH34 and *Ochrobactrum* sp. in the stationary phase.

3.3. Time-Course Biosorption Experiments of Live Biomass in Stationary and Logarithmic Phases and Dead Biomass under Environmental Conditions

In addition to investigating the cell susceptibilities in different growth phases, this study also investigated the bacterial heavy metal biosorption capacities in the same growth phases, as well as with dead biomass. As can be seen in Figure 2A, Cu^{2+} sorption by *C. metallidurans* CH34 was 10.9, and 7.7% for the cells in logarithmic and stationary phases, respectively. For the logarithmic phase, higher Cu^{2+} adsorption capacity was attained by *C. metallidurans* CH34 compared to *Ochrobactrum* sp. (Figure 2A and 2B). On the other hand, the Cr^{6+} removal by *Ochrobactrum* sp. cells in logarithmic phase was 1.4 times higher than cells in stationary phase (Figure 2D). On the other hand, the *C. metallidurans* CH34 cells in the logarithmic and stationary phases presented Cr^{6+} biosorption capacities of 16.0, and 11.5%, respectively (Figure 2C). In summary, all the results showed that microorganisms in stationary phase have reduced biosorption capacities than the ones in logarithmic phase (Figure 2).

In a previous study, Daughney also obtained better heavy metal biosorption performance by using microorganisms in logarithmic phase. In their study, they claimed that the effect of growth phase on proton adsorption is likely related to the changes in the structure and composition of the cell walls and membranes, which are directly affected by changes in the chemistry of growth medium [24]. As a result, the high abundance and availability of nutrients in the logarithmic phase stimulates the synthesis of cell components, which contains large number of molecules with heavy metal binding sites [25].

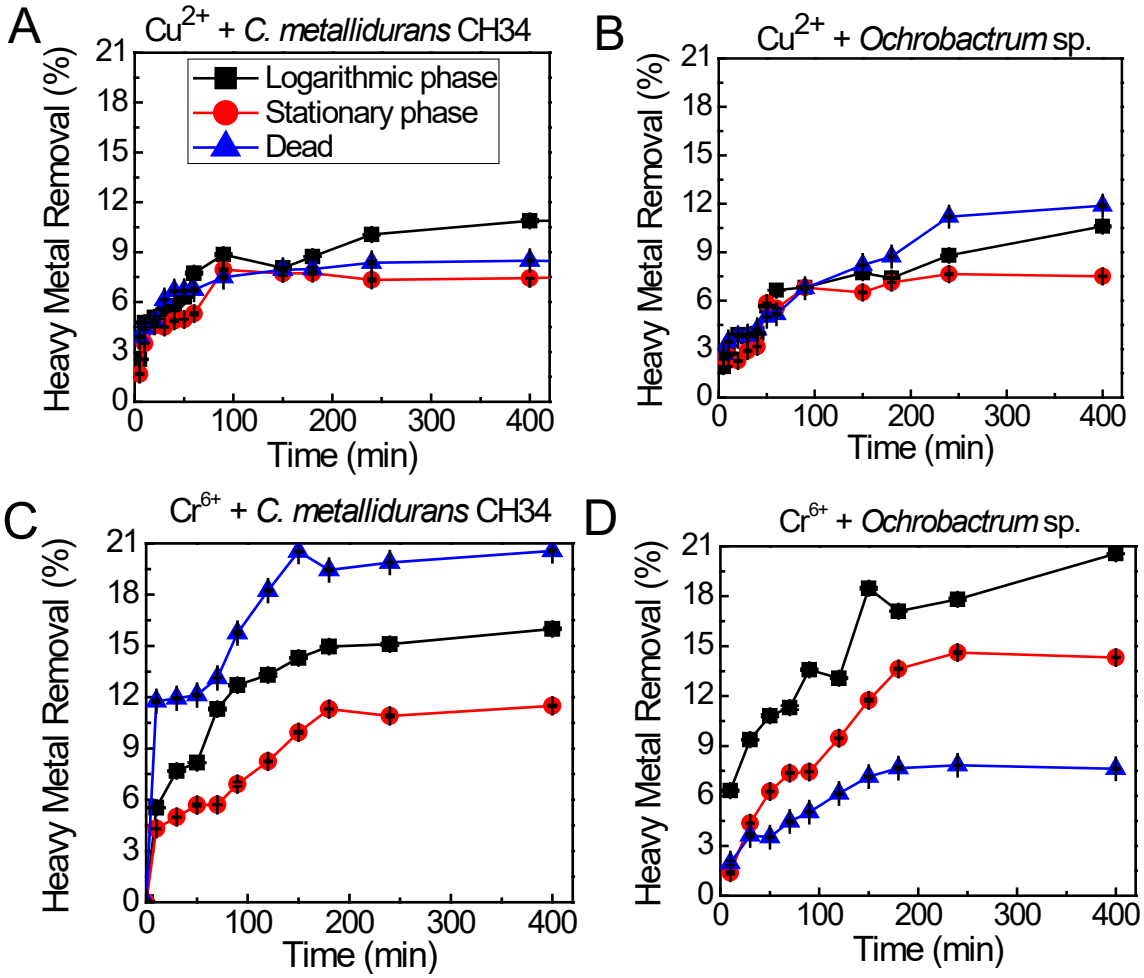

Figure 2. Equilibrium biosorption (A) *C. metallidurans* CH34 and (B) *Ochrobactrum* sp. for 100 ppm Cu^{2+}, and (C) *C. metallidurans* CH34 and (D) *Ochrobactrum* sp. for 100 ppm Cr^{6+}. All incubations were accomplished in TSM at 27°C, 80 rpm for 400 min.

In the case of dead cells, dead *Ochrobactrum* sp. and *C. metallidurans* presented higher adsorption capacities for Cu^{2+} and Cr^{6+}, respectively, than any of the other growth phases (Figure 2B and 2C). For instance, the biosorption of Cu^{2+} by dead *Ochrobactrum* sp. was approximately 12.0 and 58.0% higher than logarithmic and stationary phases, respectively (Figure 2B). Simmons and Singleton explained that intracellular components can play important roles in the heavy metal binding capacity [26]. The biosorption capacity enhancement of dead biomass could be attributed to an increasing release and exposure of intracellular binding sites caused by the heat treatment [8].

3.4. Effects of pH

Heavy metal removal capacity can be affected not only by different growth phases of the biosorbents as mentioned above, but also by certain environmental parameters, such as pH and temperature. In the present study, the effect of pH on Cu^{2+} removal is shown in Figure 3. The results indicate that the Cu^{2+} sorption capacity of *C. metallidurans* CH34 and *Ochrobactrum* sp. increased steadily with pH increase and reached a peak removal at pH 6.0, followed by a drastic decrease from pH 6.0 to 8.0. The maximum Cu^{2+} removal capacities for *C. metallidurans* CH34 (14.6%) and *Ochrobactrum* sp. (18.8%) were observed at pH 6.0. Our study is in agreement with Chen's study which also determined that the optimum pH value was 6.0 for Cu^{2+} removal by *C. metallidurans* CH34 [7].

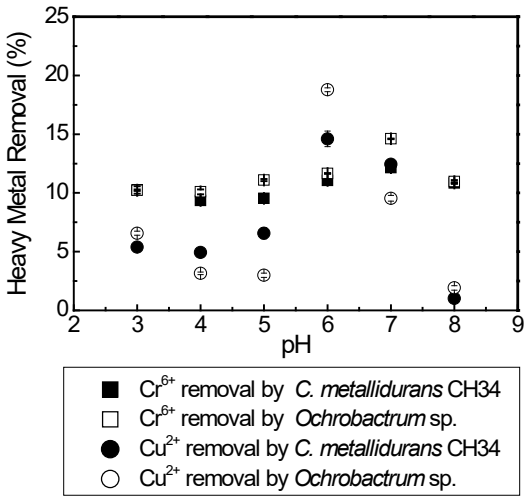

Figure 3. Effect of pH on biosorption**.** All solid symbols correspond to heavy metal removal by *C. metallidurans* CH34, while all hollow symbols correspond to heavy metal removal by *Ochrobactrum* sp.

In the case of Cr^{6+}, only a slight change was observed in the removal capacity by *Ochrobactrum* sp. and *C. metallidurans* CH34 with increasing pH. The maximum Cr^{6+} removal by *C. metallidurans* CH34 and *Ochrobactrum* sp. was achieved at pH 7 for both microorganisms, which were 12.1% and 14.6%, respectively. The main reason for

investigating the pH effect in heavy metal removal is because at low pH (pH<4) heavy metal removal efficiency is reduced. This phenomenon is caused by competition of the hydronium ions (H_3O^+) with the heavy metals for binding sites on the microbial surface [27]. In another study, it was demonstrated that if Cu^{2+} precipitation does not occur, the adsorption capacity of Cu^{2+} will not decrease at pH greater than 7 [28]. In our study, no precipitation of Cu^{2+} was observed until the pH reached 8. Therefore, the decrease in Cu^{2+} removal at pH>6 may be caused by the complexation of Cu^{2+} with some soluble organic ligands released by the microorganisms. In the case of Cr^{6+}, the optimum pH obtained for *Ochrobactrum* sp. was the same to another isolate of the same species, *Ochrobactrum intermedium* strain SDCr-5, which was pH 7 [29].

3.5. Effects of Temperature

Another environmental parameter significantly affecting heavy metal biosorption is temperature, since it is directly linked to microbial growth and metabolism. The results obtained for the removal of Cu^{2+} (Figure 4) showed that the percentage of Cu^{2+} removal decreased from 19.8% to 11.8% for *Ochrobactrum* sp. with the increase in temperature from 22^0C to 32^0C. The maximum Cu^{2+}removal value was found to be 24.4% at 37^0C.

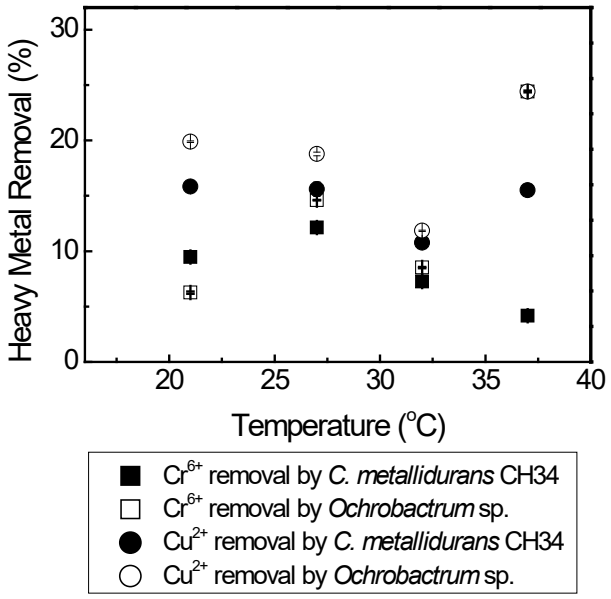

Figure 4. Temperature effect on biosorption. All solid symbols correspond to heavy metal removal by *C. metallidurans* CH34, while all hollow symbols correspond to heavy metal removal by *Ochrobactrum* sp. The experiment was conducted at pH 7.

On the other hand, the Cu^{2+} removal by *C. metallidurans* CH34 showed no significant difference ($p>0.95$) at 22°C, 27°C and 37^{0}C. In the case of Cr^{6+}, the maximum removal of heavy metal by *Ochrobactrum* sp. was obtained at 37°C (23%). In the case of *C. metallidurans* CH34, the best removal rate of Cr^{6+} occurred at 27°C, with 12.1% removal. Our results confirmed that temperature played a major role in the adsorption of heavy metal. A previous study with another strain of *Ochrobactrum* sp. showed that the optimum temperature for Cr^{6+} biosorption was 37°C, which is consistent with our results [17]. For the first time, the optimum temperature for Cu^{2+} biosorption by *Ochrobactrum* sp. was determined to be 37°C. It is worth to note that in the case of Cu^{2+} removal by *C. metallidurans* CH34, the temperature did not play a major role. A similar lack of relationship was observed for other microorganisms with copper over a temperature range of 25°C to 55°C [30].

3.6. Langmuir Isotherms of Biosorption

After determining the effects of temperature and pH in the adsorption of Cu^{2+} and Cr^{6+}, adsorption capacities were modeled using the Langmuir model. As seen in Table 2, the Cu^{2+} removal capacity of *C. metallidurans* CH34 was 86.78 mg/g by dead cells and cells in logarithmic phase, which is about 2.2 times higher than cells in stationary phase. The Q_{max} value for Cr^{6+} biosorption of dead *C. metallidurans* CH34 was 47.79 mg/g, indicating a higher Cr^{6+} biosorption capacity compared to both cells in logarithmic and stationary phases. Furthermore, when the results obtained for Cu^{2+} and Cr^{6+} removal by the same strain were compared, *C. metallidurans* CH34 always showed higher biosorption capacity for Cu^{2+} in a range of 1.8 to 2.6 times higher than that for Cr^{6+}. *Ochrobactrum* sp. also presented a similar adsorption trend to *C. metallidurans* CH34 for Cu^{2+}. These results suggested that Cu^{2+} has greater affinity to the binding sites presented on the surface of *C. metallidurans* CH34 and *Ochrobactrum* sp. than Cr^{6+}. Previous reports also suggested that metal sorption increased with increasing valence and atomic number [31]. Therefore, as expected, in this study, the sorption capacity for $^{29}Cu^{2+}$ was higher than for $^{24}Cr^{6+}$, which is in agreement with previous reports that also demonstrated this sorption preference [32].

Table 2. Langmuir model estimated parameters for *C. metallidurans* CH34 and *Ochrobactrum sp*. in different bacterial growth phases.

Heavy metals	Bacterial growth phases	Bacterial strains as absorbents	Estimated parameters for the Langmuir isotherm		
			K_d	Q_{max}(mg/g)	R^2
Cu^{2+}	logarithmic	*C. metallidurans* CH34	0.0067	86.78	0.93
		Ochrobactrum sp.	0.0062	57.68	0.95
	stationary	*C. metallidurans* CH34	0.0025	38.72	0.97
		Ochrobactrum sp.	0.0039	49.93	0.96
	dead	*C. metallidurans* CH34	0.0080	86.78	0.97
		Ochrobactrum sp.	0.0046	75.14	0.96
Cr^{6+}	logarithmic	*C. metallidurans* CH34	0.0311	32.63	0.90

	Ochrobactrum sp.	0.0116	51.96	0.96
stationary	*C. metallidurans* CH34	0.0229	20.48	0.99
	Ochrobactrum sp.	0.0132	23.61	0.95
dead	*C. metallidurans* CH34	0.0288	47.79	0.96
	Ochrobactrum sp.	0.0173	10.94	0.95

K_d: Langmuir constant; Q_{max}: maximum adsorption capacity;R^2: regression coefficient

In this study, the dead biomass Langmuir isotherm model presented a R^2 value higher than the logarithmic and stationary phases for the biosorption of Cu^{2+}. This indicates that the biosorption of Cu^{2+} onto dead cells of *C. metallidurans* CH34 and *Ochrobactrum* sp. were likely to be of a monolayer type of sorption. Similar results were reported by a number of studies on adsorption of heavy metals using dead cells [3].

However, in one special case, dead *Ochrobactrum* sp. was not as effective as live biomass in the removal of Cr^{6+}. The adsorption capacity of Cr^{6+} for *Ochrobactrum* sp. presented a Q_{max} of 51.96 mg/g and 23.61 mg/g for logarithmic and stationary phases, respectively; while the dead biomass presented the lowest Q_{max} (10.94 mg/g). Therefore, not all dead biomass from microorganisms present a good removal capacity.

3.7. Cu^{2+} and Cr^{6+} Adsorption Competition Assays under Environmental and Optimum Conditions

After investigating the single metal biosorption, the next step was to understand the heavy metal binding preference by each microorganism. The results of the competitive biosorption assays with *Ochrobactrum* sp. indicated that Cu^{2+}removal increased with the decreasing ratio of Cr^{6+}/Cu^{2+} (Figure 5). It is worth noting that the percentage of Cu^{2+} removal was always 0.5-2 times higher than the removal of Cr^{6+} under both environmental and optimum growth conditions with different ratios of Cr^{6+}/Cu^{2+}. Moreover, the optimum microbial growth condition was an important parameter which

led to 10%-47% higher removal of Cu^{2+} and Cr^{6+} than the environmental condition. The optimum temperatures for heavy metal removal were 37ºC and 27ºC for *Ochrobactrum* sp. and *C. metallidurans* CH34, respectively. For both microorganisms, the optimum pH was 6 for Cu^{2+} removal, while the best pH for Cr^{6+} biosorption was 7. The results for the Langmuir isotherm model parameters also showed similar results (Table S1). These results not only demonstrated the importance of the environmental parameters like pH and temperature in the removal of heavy metal by microbial biomass, but also allowed to determine the heavy metal removal efficiencies of these two microorganisms under optimum and non-optimum conditions for each microorganism.

When comparing the results of the environmental conditions of a single metal with a mixture of Cu^{2+} and Cr^{6+}, the removal of Cu^{2+} by *Ochrobactrum* sp. decreased by 9% with the highest ratio of Cu^{2+} in the presence of Cr^{6+}, the competing metal ion. A much lower removal was achieved with a higher ratio of Cr^{6+}. Similar results were observed for *C. metallidurans* CH34, which presented a lower removal capacity of Cu^{2+} and Cr^{6+} in the heavy metal mixture than in a solution with a single metal.

The mechanism of multi-metal ions uptake by microorganisms is quite complex. Competition for the surface binding sites among the different metal-ions will occur and will partially depend on the metal ionic characteristics [33]. There are also possible interactive effects of different species in solution and potential interactions with the biomass surface [34]. This antagonistic action is due to the competition for the same adsorption sites on the cells. Another widely reported explanation for this antagonistic effect is the screening effect [35]. The screening effect may arise as the concentration of the biomass increases, leading to the decrease in attraction between electron and nucleus,

and then rendering some binding sites partially hindered to metal ions. Hence, the specific metal uptake capacity will decrease. Additionally, as depicted in Figure 5, the uptake percentage of Cu^{2+} increased with the increasing of Cu^{2+} concentration. This result suggests that the Cu^{2+} binding sites are more abundant or the cell surface have preferential binding sites for Cu^{2+} rather than Cr^{6+}, which enhanced the uptake of Cu^{2+} metal ions [36].

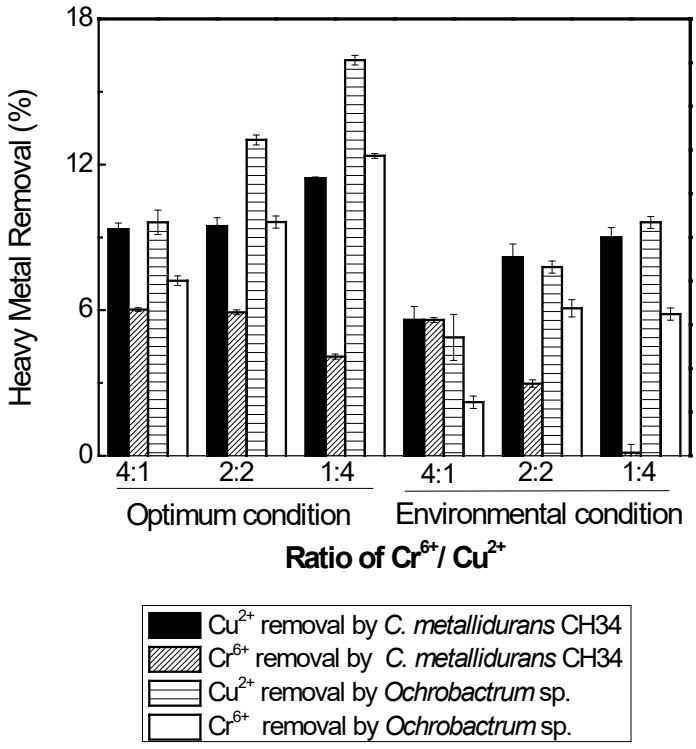

Figure 5. Heavy metal removal in the presence of different ratios (4:1, 2:2, and 1:4) of Cu^{2+} and Cr^{6+}.

3.8. Biosorption Mechanisms

The initial confirmation of heavy metal adsorption by *C. metallidurans* CH34 and *Ochrobactrum* sp. was obtained with EDS (Supporting information Figure S2). Among various proposed removal mechanisms, ion exchange was thought to be one of the most important processes for heavy metal removal by bacterial biomass. For instance, Tunali reported that ion exchange was involved in the Pb^{2+} and Cu^{2+} removal by *Bacillus* sp.

[37]. Moreover, Srivastava and collaborators revealed through TEM and EDS analyses that Cr^{3+} was adsorbed and later up taken inside the cells [38].

Furthermore, heavy metal removal by surface complexation with the cell membrane and cell wall functional groups is also widely reported to be an important mechanism for biosorption [39]. Therefore, FTIR was investigated to assess the contribution of the functional groups in the cell membrane (Figure S3). The summary of the possible metal binding sites is presented in Table 3. The results of the FTIR spectra of the live biomass without interaction with heavy metal showed five distinct peaks. The changes in the corresponding functional groups for Cu^{2+} and Cr^{6+} biosorption observed in our results are identical to previous studies with other microorganisms [40]. They concluded that the main function groups responsible for biosorption of heavy metals in bacteria are carboxylic, hydroxyl and amino groups [41].

When comparing the heat-treated biomass with the live biomass, multiple peaks of low intensity for the C=O functional groups coming from proteins between 1500 cm^{-1} and 1800 cm^{-1} were observed for heat-treated biomass, as opposed to one to two peaks of high intensity for the live biomass. This suggested that more binding sites became available after the thermal treatment and that the cellular membranes were no longer intact, which increased the biosorption capacity of the dead cells.

Table 3. The FTIR band assignments and the possible metal binding sites in both bacteria.

Wave number (cm^{-1})			Assignment	Probable sites for Cu^{2+} and Cr^{6+} interaction with live *Ochrobactrum* sp. and *C. metallidurans* CH34	Probable sites for Cu^{2+} and Cr^{6+} interaction with dead *Ochrobactrum* sp. and *C. metallidurans* CH34
Logarithmic phase	Stationary phase	Dead			
1440	1440	1440	C-H	Binding to lipoprotein	Binding to lipoprotein
1651	1651	1620	C=O	Interaction with protein	Interaction with protein
1710	1710	1703	C=O	Interaction with protein	Interaction with protein
-	-	1728	C=O and COOH	-	Interaction with protein
2940	2940	2940	C-H	-	-

3327	3327	3327	N-H_2 and O-H	Interaction with polysaccharide and proteins	Interaction with polysaccharide and proteins

4. Conclusion

This study demonstrated that *C. metallidurans* CH34 and *Ochrobactrum* sp. have an excellent tolerance to Cu^{2+} and Cr^{6+}. The results about biosorption showed that adsorption of Cu^{2+} and Cr^{6+} microorganisms are largely dependent on growth phase. Environmental factors, such as pH and temperature, also affected the adsorption capacity significantly. The optimized condition of pH, temperature and growth phase led to 10% to 47% higher removal of Cu^{2+} and Cr^{6+} than environmental conditions. Therefore, these results suggest that these microorganisms can be good candidates for cleaning up environments contaminated with both $Cu^{2+}$and Cr^{6+}, as long as the proper environmental conditions for their heavy metal removal is met.

Acknowledgements

We would like to thank the Texas Association of Environmental Professionals for the fellowship given to Jinjing Fan during the execution of this study. We also would like to thank Dr. Solange Sakata for providing the *Ochrobactrum* sp. isolate for this investigation.

References

[1] V.B.H. Dang, H.D. Doan, T. Dang-Vu, A. Lohi, Equilibrium and kinetics of biosorption of cadmium(II) and copper(II) ions by wheat straw, Bioresource Technology, 100 (2009) 211-219.

[2] Y.L.F. Musico, C.M. Santos, M.L.P. Dalida, D.F. Rodrigues, Improved removal of lead(ii) from water using a polymer-based graphene oxide nanocomposite, Journal of Materials Chemistry A, 1 (2013) 3789-3796.

[3] H. Li, Y. Lin, W. Guan, J. Chang, L. Xu, J. Guo, G. Wei, Biosorption of Zn(II) by live and dead cells of Streptomyces ciscaucasicus strain CCNWHX 72-14, Journal of hazardous materials, 179 (2010) 151-159.
[4] J.L. Wang, C. Chen, Biosorbents for heavy metals removal and their future, Biotechnology Advances, 27 (2009) 195-226.
[5] J. Tangaromsuk, P. Pokethitiyook, M. Kruatrachue, E.S. Upatham, Cadmium biosorption by Sphingomonas paucimobilis biomass, Bioresource technology, 85 (2002) 103-105.
[6] J.S. Chang, R. Law, C.C. Chang, Biosorption of lead, copper and cadmium by biomass of Pseudomonas aeruginosa PU21, Water Research, 31 (1997) 1651-1658.
[7] W.M. Chen, C.H. Wu, E.K. James, J.S. Chang, Metal biosorption capability of Cupriavidus taiwanensis and its effects on heavy metal removal by nodulated Mimosa pudica, Journal of hazardous materials, 151 (2008) 364-371.
[8] R.M. Gabr, S.H.A. Hassan, A.A.M. Shoreit, Biosorption of lead and nickel by living and non-living cells of Pseudomonas aeruginosa ASU 6a, International Biodeterioration & Biodegradation, 62 (2008) 195-203.
[9] M.L. Ledrich, S. Stemmler, P. Laval-Gilly, L. Foucaud, J. Falla, Precipitation of silver-thiosulfate complex and immobilization of silver by Cupriavidus metallidurans CH34, Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, 18 (2005) 643-650.
[10] J. Goris, P. De Vos, T. Coenye, B. Hoste, D. Janssens, H. Brim, L. Diels, M. Mergeay, K. Kersters, P. Vandamme, Classification of metal-resistant bacteria from industrial biotopes as Ralstonia campinensis sp. nov., Ralstonia metallidurans sp. nov. and Ralstonia basilensis Steinle et al. 1998 emend, International journal of systematic and evolutionary microbiology, 51 (2001) 1773-1782.
[11] M. Mergeay, D. Nies, H.G. Schlegel, J. Gerits, P. Charles, F. Vangijsegem, Alcaligenes-Eutrophus Ch34 Is a Facultative Chemolithotroph with Plasmid-Bound Resistance to Heavy-Metals, Journal of bacteriology, 162 (1985) 328-334.
[12] K.V. Lakshmi, C. Sasikala, V. Ramana Ch, Rhodoplanes pokkaliisoli sp. nov., a phototrophic alphaproteobacterium isolated from a waterlogged brackish paddy

soil, International journal of systematic and evolutionary microbiology, 59 (2009) 2153-2157.
[13] G.M. Balestra, I.J. Misaghi, Increasing the efficiency of the plate counting method for estimating bacterial diversity, Journal of Microbiological Methods, 30 (1997) 111-117.
[14] M.I. Kefala, A.I. Zouboulis, K.A. Matis, Biosorption of cadmium ions by Actinomycetes and separation by flotation, Environmental Pollution, 104 (1999) 283-293.
[15] G.M. Teitzel, M.R. Parsek, Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa, Applied and environmental microbiology, 69 (2003) 2313-2320.
[16] I.E. Mejias Carpio, C.M. Santos, X. Wei, D.F. Rodrigues, Toxicity of a polymer-graphene oxide composite against bacterial planktonic cells, biofilms, and mammalian cells, Nanoscale, 4 (2012) 4746-4756.
[17] M. Faisal, S. Hasnain, Comparative study of Cr(VI) uptake and reduction in industrial effluent by Ochrobactrum intermedium and Brevibacterium sp., Biotechnology Letters, 26 (2004) 1623-1628.
[18] Z.A. Zakaria, Z. Zakaria, S. Surif, W.A. Ahmad, Hexavalent chromium reduction by Acinetobacter haemolyticus isolated from heavy-metal contaminated wastewater, Journal of hazardous materials, 146 (2007) 30-38.
[19] J.P. Ruparelia, A.K. Chatterjee, S.P. Duttagupta, S. Mukherji, Strain specificity in antimicrobial activity of silver and copper nanoparticles, Acta biomaterialia, 4 (2008) 707-716.
[20] G. Ozdemir, N. Ceyhan, T. Ozturk, F. Akirmak, T. Cosar, Biosorption of chromium(VI), cadmium(II) and copper(II) by Pantoea sp TEM, Chemical Engineering Journal, 102 (2004) 249-253.
[21] I. Ahmad, M.I. Ansari, F. Aqil, Biosorption of Ni, Cr and Cd by metal tolerant Aspergillus niger and Penicillium sp. using single and multi-metal solution, Indian journal of experimental biology, 44 (2006) 73-76.
[22] J.L. Meers, D.W. Tempest, The influence of growth-limiting substrate and medium NaCl concentration on the synthesis of magnesium-binding sites in the

walls of Bacillus subtilis var. niger, Journal of general microbiology, 63 (1970) 325-331.
[23] S. Kjelleberg, M. Hermansson, P. Marden, G.W. Jones, The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment, Annual review of microbiology, 41 (1987) 25-49.
[24] C.J. Daughney, D.A. Fowle, D.E. Fortin, The effect of growth phase on proton and metal adsorption by Bacillus subtilis, Geochimica Et Cosmochimica Acta, 65 (2001) 1025-1035.
[25] R.J.C. Mclean, D. Beauchemin, L. Clapham, T.J. Beveridge, Metal-Binding Characteristics of the Gamma-Glutamyl Capsular Polymer of Bacillus-Licheniformis Atcc-9945, Applied and Environmental Microbiology, 56 (1990) 3671-3677.
[26] P. Simmons, I. Singleton, A method to increase silver biosorption by an industrial strain of Saccharomyces cerevisiae, Applied Microbiology and Biotechnology, 45 (1996) 278-285.
[27] S. Karthikeyan, P.R. Palaniappan, S. Sabhanayakam, Influence of pH and water hardness upon nickel accumulation in edible fish Cirrhinus mrigala, Journal of environmental biology / Academy of Environmental Biology, India, 28 (2007) 489-492.
[28] C.-p. Huang, C.-p. Huang, A.L. Morehart, The removal of Cu(II) from dilute aqueous solutions by Saccharomyces cerevisiae, Water Research, 24 (1990) 433-439.
[29] S. Sultan, S. Hasnain, Reduction of toxic hexavalent chromium by Ochrobactrum intermedium strain SDCr-5 stimulated by heavy metals, Bioresource Technology, 98 (2007) 340-344.
[30] W.M. Antunes, A.S. Luna, C.A. Henriques, A.C.A. da Costa, An evaluation of copper biosorption by a brown seaweed under optimized conditions, Electronic Journal of Biotechnology, 6 (2003) 174-184.
[31] A. Ozer, D. Ozer, Comparative study of the biosorption of Pb(II), Ni(II) and Cr(VI) ions onto S-cerevisiae: determination of biosorption heats, Journal of hazardous materials, 100 (2003) 219-229.

[32] B. Nasernejad, T.E. Zadeh, B.B. Pour, M.E. Bygi, A. Zamani, Camparison for biosorption modeling of heavy metals (Cr(III), Cu(II), Zn(II)) adsorption from wastewater by carrot residues, Process Biochemistry, 40 (2005) 1319-1322.
[33] S. Tunali, T. Akar, Zn(II) biosorption properties of Botrytis cinerea biomass, Journal of hazardous materials, 131 (2006) 137-145.
[34] Z. Aksu, G. Donmez, Binary biosorption of cadmium(II) and nickel(II) onto dried Chlorella vulgaris: Co-ion effect on mono-component isotherm parameters, Process Biochemistry, 41 (2006) 860-868.
[35] Y. Sag, T. Kutsal, The selective biosorption of chromium(VI) and copper(II) ions from binary metal mixtures by R-arrhizus, Process Biochemistry, 31 (1996) 561-572.
[36] F. Pagnanelli, M. Trifoni, F. Beolchini, A. Esposito, L. Toro, F. Veglio, Equilibrium biosorption studies in single and multi-metal systems, Process Biochemistry, 37 (2001) 115-124.
[37] S. Tunali, A. Cabuk, T. Akar, Removal of lead and copper ions from aqueous solutions by bacterial strain isolated from soil, Chemical Engineering Journal, 115 (2006) 203-211.
[38] S. Srivastava, I.S. Thakur, Biosorption potency of Aspergillus niger for removal of chromium (VI), Current Microbiology, 53 (2006) 232-237.
[39] C. Quintelas, B. Femandes, J. Castro, H. Figueiredo, T. Tavares, Biosorption of Cr(VI) by a Bacillus coagulans biofilm supported on granular activated carbon (GAC), Chemical Engineering Journal, 136 (2008) 195-203.
[40] I. Kiran, T. Akar, S. Tunali, Biosorption of Pb(II) and Cu(II) from aqueous solutions by pretreated biomass of Neurospora crassa, Process Biochemistry, 40 (2005) 3550-3558.
[41] T. Akar, S. Tunali, Biosorption performance of Botrytis cinerea fungal by-products for removal of Cd(II) and Cu(II) ions from aqueous solutions, Minerals Engineering, 18 (2005) 1099-1109.